

1                   CANCEROUS DISEASE MODIFYING ANTIBODIES

2

3   CROSS REFERENCE TO RELATED APPLICATIONS

4           This application is a continuation of serial number 10/348,231, filed on January 21,  
5   2003, the contents of which are herein incorporated by reference.

6

7   FIELD OF THE INVENTION

8           This invention relates to the isolation and production of cancerous disease  
9   modifying antibodies (CDMAB) and to the use of these CDMAB in therapeutic and  
10   diagnostic processes, optionally in combination with one or more chemotherapeutic agents.  
11   The invention further relates to binding assays which utilize the CDMABs of the instant  
12   invention.

13

14   BACKGROUND OF THE INVENTION

15           Each individual who presents with cancer is unique and has a cancer that is as  
16   different from other cancers as that person's identity. Despite this, current therapy treats  
17   all patients with the same type of cancer, at the same stage, in the same way. At least 30%  
18   of these patients will fail the first line therapy, thus leading to further rounds of treatment  
19   and the increased probability of treatment failure, metastases, and ultimately, death. A  
20   superior approach to treatment would be the customization of therapy for the particular  
21   individual. The only current therapy which lends itself to customization is surgery.

McHale & Slavin, P.A. 2056.027

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Express Mail No. EV303518319US

1     Chemotherapy and radiation treatment can not be tailored to the patient, and surgery by  
2     itself, in most cases is inadequate for producing cures.

3             With the advent of monoclonal antibodies, the possibility of developing methods  
4     for customized therapy became more realistic since each antibody can be directed to a  
5     single epitope. Furthermore, it is possible to produce a combination of antibodies that are  
6     directed to the constellation of epitopes that uniquely define a particular individual's  
7     tumor.

8             Having recognized that a significant difference between cancerous and normal cells  
9     is that cancerous cells contain antigens that are specific to transformed cells, the scientific  
10    community has long held that monoclonal antibodies can be designed to specifically target  
11    transformed cells by binding specifically to these cancer antigens; thus giving rise to the  
12    belief that monoclonal antibodies can serve as "Magic Bullets" to eliminate cancer cells.

13            Monoclonal antibodies isolated in accordance with the teachings of the instantly  
14    disclosed invention have been shown to modify the cancerous disease process in a manner  
15    which is beneficial to the patient, for example by reducing the tumor burden, and will  
16    variously be referred to herein as cancerous disease modifying antibodies (CDMAB) or  
17    "anti-cancer" antibodies.

18            At the present time, the cancer patient usually has few options of treatment. The  
19    regimented approach to cancer therapy has produced improvements in global survival and  
20    morbidity rates. However, to the particular individual, these improved statistics do not  
21    necessarily correlate with an improvement in their personal situation.

1           Thus, if a methodology was put forth which enabled the practitioner to treat each  
2 tumor independently of other patients in the same cohort, this would permit the unique  
3 approach of tailoring therapy to just that one person. Such a course of therapy would,  
4 ideally, increase the rate of cures, and produce better outcomes, thereby satisfying a long-  
5 felt need.

6           Historically, the use of polyclonal antibodies has been used with limited success in  
7 the treatment of human cancers. Lymphomas and leukemias have been treated with human  
8 plasma, but there were few prolonged remission or responses. Furthermore, there was a  
9 lack of reproducibility and there was no additional benefit compared to chemotherapy.  
10 Solid tumors such as breast cancers, melanomas and renal cell carcinomas have also been  
11 treated with human blood, chimpanzee serum, human plasma and horse serum with  
12 correspondingly unpredictable and ineffective results.

13           There have been many clinical trials of monoclonal antibodies for solid tumors. In  
14 the 1980s there were at least four clinical trials for human breast cancer which produced  
15 only one responder from at least 47 patients using antibodies against specific antigens or  
16 based on tissue selectivity. It was not until 1998 that there was a successful clinical trial  
17 using a humanized anti-her 2 antibody in combination with Cisplatin. In this trial 37  
18 patients were assessed for responses of which about a quarter had a partial response rate  
19 and another half had minor or stable disease progression.

20           The clinical trials investigating colorectal cancer involve antibodies against both  
21 glycoprotein and glycolipid targets. Antibodies such as 17-1A, which has some specificity

1 for adenocarcinomas, had undergone Phase 2 clinical trials in over 60 patients with only  
2 one patient having a partial response. In other trials, use of 17-1A produced only one  
3 complete response and two minor responses among 52 patients in protocols using  
4 additional cyclophosphamide. Other trials involving 17-1A yielded results that were  
5 similar. The use of a humanized murine monoclonal antibody initially approved for  
6 imaging also did not produce tumor regression. To date there has not been an antibody  
7 that has been effective for colorectal cancer. Likewise there have been equally poor results  
8 for lung cancer, brain cancers, ovarian cancers, pancreatic cancer, prostate cancer, and  
9 stomach cancer. There has been some limited success in the use of anti-GD3 monoclonal  
10 antibody for melanoma. Thus, it can be seen that despite successful small animal studies  
11 that are a prerequisite for human clinical trials, the antibodies that have been tested have  
12 been for the most part ineffective.

13

#### 14 PRIOR PATENTS

15 U.S. Patent No. 5,750,102 discloses a process wherein cells from a patient's tumor  
16 are transfected with MHC genes which may be cloned from cells or tissue from the patient.  
17 These transfected cells are then used to vaccinate the patient.

18 U.S. Patent No. 4,861,581 discloses a process comprising the steps of obtaining  
19 monoclonal antibodies that are specific to an internal cellular component of neoplastic and  
20 normal cells of the mammal but not to external components, labeling the monoclonal  
21 antibody, contacting the labeled antibody with tissue of a mammal that has received

1 therapy to kill neoplastic cells, and determining the effectiveness of therapy by measuring  
2 the binding of the labeled antibody to the internal cellular component of the degenerating  
3 neoplastic cells. In preparing antibodies directed to human intracellular antigens, the  
4 patentee recognizes that malignant cells represent a convenient source of such antigens.

5 U.S. Patent No. 5,171,665 provides a novel antibody and method for its production.  
6 Specifically, the patent teaches formation of a monoclonal antibody which has the property  
7 of binding strongly to a protein antigen associated with human tumors, e.g. those of the  
8 colon and lung, while binding to normal cells to a much lesser degree.

9 U.S. Patent No. 5,484,596 provides a method of cancer therapy comprising  
10 surgically removing tumor tissue from a human cancer patient, treating the tumor tissue to  
11 obtain tumor cells, irradiating the tumor cells to be viable but non-tumorigenic, and using  
12 these cells to prepare a vaccine for the patient capable of inhibiting recurrence of the  
13 primary tumor while simultaneously inhibiting metastases. The patent teaches the  
14 development of monoclonal antibodies which are reactive with surface antigens of tumor  
15 cells. As set forth at col. 4, lines 45 et seq., the patentees utilize autochthonous tumor cells  
16 in the development of monoclonal antibodies expressing active specific immunotherapy in  
17 human neoplasia.

18 U.S. Patent No. 5,693,763 teaches a glycoprotein antigen characteristic of human  
19 carcinomas and not dependent upon the epithelial tissue of origin.

20 U.S. Patent No. 5,783,186 is drawn to Anti-Her2 antibodies which induce apoptosis  
21 in Her2 expressing cells, hybridoma cell lines producing the antibodies, methods of

1     treating cancer using the antibodies and pharmaceutical compositions including said  
2     antibodies.

3             U.S. Patent No. 5,849,876 describes new hybridoma cell lines for the production of  
4     monoclonal antibodies to mucin antigens purified from tumor and non-tumor tissue  
5     sources.

6             U.S. Patent No. 5,869,268 is drawn to a method for generating a human  
7     lymphocyte producing an antibody specific to a desired antigen, a method for producing a  
8     monoclonal antibody, as well as monoclonal antibodies produced by the method. The  
9     patent is particularly drawn to the production of an anti-HD human monoclonal antibody  
10    useful for the diagnosis and treatment of cancers.

11            U.S. Patent No. 5,869,045 relates to antibodies, antibody fragments, antibody  
12    conjugates and single chain immunotoxins reactive with human carcinoma cells. The  
13    mechanism by which these antibodies function is two-fold, in that the molecules are  
14    reactive with cell membrane antigens present on the surface of human carcinomas, and  
15    further in that the antibodies have the ability to internalize within the carcinoma cells,  
16    subsequent to binding, making them especially useful for forming antibody-drug and  
17    antibody-toxin conjugates. In their unmodified form the antibodies also manifest cytotoxic  
18    properties at specific concentrations.

19            U.S. Patent No. 5,780,033 discloses the use of autoantibodies for tumor therapy and  
20    prophylaxis. However, this antibody is an antinuclear autoantibody from an aged mammal.  
21    In this case, the autoantibody is said to be one type of natural antibody found in the

immune system. Because the autoantibody comes from "an aged mammal", there is no requirement that the autoantibody actually comes from the patient being treated. In addition the patent discloses natural and monoclonal antinuclear autoantibody from an aged mammal, and a hybridoma cell line producing a monoclonal antinuclear autoantibody.

## SUMMARY OF THE INVENTION

The instant inventors have previously been awarded U.S. Patent 6,180,357, entitled "Individualized Patient Specific Anti-Cancer Antibodies" directed to a process for selecting individually customized anti-cancer antibodies which are useful in treating a cancerous disease.

This application utilizes the method for producing patient specific anti-cancer antibodies as taught in the '357 patent for isolating hybridoma cell lines which encode for cancerous disease modifying monoclonal antibodies. These antibodies can be made specifically for one tumor and thus make possible the customization of cancer therapy. Within the context of this application, anti-cancer antibodies having either cell-killing (cytotoxic) or cell-growth inhibiting (cytostatic) properties will hereafter be referred to as cytotoxic. These antibodies can be used in aid of staging and diagnosis of a cancer, and can be used to treat tumor metastases.

The prospect of individualized anti-cancer treatment will bring about a change in the way a patient is managed. A likely clinical scenario is that a tumor sample is obtained

1 at the time of presentation, and banked. From this sample, the tumor can be typed from a  
2 panel of pre-existing cancerous disease modifying antibodies. The patient will be  
3 conventionally staged but the available antibodies can be of use in further staging the  
4 patient. The patient can be treated immediately with the existing antibodies, and a panel of  
5 antibodies specific to the tumor can be produced either using the methods outlined herein  
6 or through the use of phage display libraries in conjunction with the screening methods  
7 herein disclosed. All the antibodies generated will be added to the library of anti-cancer  
8 antibodies since there is a possibility that other tumors can bear some of the same epitopes  
9 as the one that is being treated. The antibodies produced according to this method  
10 may be useful to treat cancerous disease in any number of patients who have cancers that  
11 bind to these antibodies.

12 In addition to anti-cancer antibodies, the patient can elect to receive the currently  
13 recommended therapies as part of a multi-modal regimen of treatment. The fact that the  
14 antibodies isolated via the present methodology are relatively non-toxic to non-cancerous  
15 cells allows for combinations of antibodies at high doses to be used, either alone, or in  
16 conjunction with conventional therapy. The high therapeutic index will also permit re-  
17 treatment on a short time scale that should decrease the likelihood of emergence of  
18 treatment resistant cells.

19 Furthermore, it is within the purview of this invention to conjugate standard  
20 chemotherapeutic modalities, e.g. radionuclides, with the CDMABs of the instant  
21 invention, thereby focusing the use of said chemotherapeutics.



1           If the patient is refractory to the initial course of therapy or metastases develop, the  
2   process of generating specific antibodies to the tumor can be repeated for re-treatment.  
3   Furthermore, the anti-cancer antibodies can be conjugated to red blood cells obtained from  
4   that patient and re-infused for treatment of metastases. There have been few effective  
5   treatments for metastatic cancer and metastases usually portend a poor outcome resulting  
6   in death. However, metastatic cancers are usually well vascularized and the delivery of  
7   anti-cancer antibodies by red blood cells can have the effect of concentrating the antibodies  
8   at the site of the tumor. Even prior to metastases, most cancer cells are dependent on the  
9   host's blood supply for their survival and anti-cancer antibody conjugated to red blood  
10   cells can be effective against *in situ* tumors as well. Alternatively, the antibodies may be  
11   conjugated to other hematogenous cells, e.g. lymphocytes, macrophages, monocytes,  
12   natural killer cells, etc.

13           There are five classes of antibodies and each is associated with a function that is  
14   conferred by its heavy chain. It is generally thought that cancer cell killing by naked  
15   antibodies are mediated either through antibody dependent cellular cytotoxicity or  
16   complement dependent cytotoxicity. For example murine IgM and IgG2a antibodies can  
17   activate human complement by binding the C-1 component of the complement system  
18   thereby activating the classical pathway of complement activation which can lead to tumor  
19   lysis. For human antibodies the most effective complement activating antibodies are  
20   generally IgM and IgG1. Murine antibodies of the IgG2a and IgG3 isotype are effective at  
21   recruiting cytotoxic cells that have Fc receptors which will lead to cell killing by

1 monocytes, macrophages, granulocytes and certain lymphocytes. Human antibodies of  
2 both the IgG1 and IgG3 isotype mediate ADCC.

3 Another possible mechanism of antibody mediated cancer killing may be through  
4 the use of antibodies that function to catalyze the hydrolysis of various chemical bonds in  
5 the cell membrane and its associated glycoproteins or glycolipids, so-called catalytic  
6 antibodies.

7 There are two additional mechanisms of antibody mediated cancer cell killing  
8 which are more widely accepted. The first is the use of antibodies as a vaccine to induce  
9 the body to produce an immune response against the putative cancer antigen that resides on  
10 the tumor cell. The second is the use of antibodies to target growth receptors and interfere  
11 with their function or to down regulate that receptor so that effectively its function is lost.

12 Accordingly, it is an objective of the invention to utilize a method for producing  
13 cancerous disease modifying antibodies from cells derived from a particular individual  
14 which are cytotoxic with respect to cancer cells while simultaneously being relatively non-  
15 toxic to non-cancerous cells, in order to isolate hybridoma cell lines and the corresponding  
16 isolated monoclonal antibodies and antigen binding fragments thereof for which said  
17 hybridoma cell lines are encoded.

18 It is an additional objective of the invention to teach cancerous disease modifying  
19 antibodies and antigen binding fragments thereof.

1           It is a further objective of the instant invention to produce cancerous disease  
2   modifying antibodies whose cytotoxicity is mediated through antibody dependent cellular  
3   toxicity.

4           It is yet an additional objective of the instant invention to produce cancerous  
5   disease modifying antibodies whose cytotoxicity is mediated through complement  
6   dependent cellular toxicity.

7           It is still a further objective of the instant invention to produce cancerous disease  
8   modifying antibodies whose cytotoxicity is a function of their ability to catalyze hydrolysis  
9   of cellular chemical bonds.

10          A still further objective of the instant invention is to produce cancerous disease  
11   modifying antibodies which are useful for in a binding assay for diagnosis, prognosis, and  
12   monitoring of cancer.

13          Other objects and advantages of this invention will become apparent from the  
14   following description wherein are set forth, by way of illustration and example, certain  
15   embodiments of this invention.

16

#### 17   BRIEF DESCRIPTION OF THE FIGURES

18   Figure 1 includes representative FACS histograms of 1A245.6 antibodies, isotype control  
19   antibodies for both antibodies, anti-EGFR antibodies directed against several cancer cell  
20   lines and non-cancer cells;

1 Figure 2 includes representative FACS histograms of 7BD-33-11A antibodies, isotype  
2 control antibodies for 1A245.6, anti-EGFR antibodies, isotype control antibodies for anti-  
3 EGFR directed against several cancer cell lines and non-cancer cells;  
4 Figure 3 includes representative FACS histograms of 11BD-2E11-2 antibodies, isotype  
5 control antibodies for both antibodies, anti-EGFR antibodies directed against several  
6 cancer cell lines and non-cancer cells;  
7 Figure 4 is a graphical analysis of tumor volume over time with respect to particular  
8 antibody treatment;  
9 Figure 5 is a graphical analysis of antibody effect on MB231 Human Breast Cancer tumor  
10 volume over time;  
11 Figure 6 is a graphical analysis quantifying percent survival over time relative to antibody  
12 therapy.

13

14 EXAMPLE 1

15 Hybridomas Production – Hybridoma Cell Line 7BD-33-11A, 1A245.6, 11BD-2E11-2

16 Hybridomas:

17 The hybridoma cell lines 7BD-33-11A and 1A245.6 were deposited, in accordance  
18 with the Budapest Treaty, with the American Type Culture Collection, 10801 University  
19 Blvd., Manassas, VA 20110-2209 on January 8, 2003, under Accession Number PTA-  
20 4890 and PTA-4889, respectively. In accordance with 37 CFR 1.808, the depositors assure

1 that all restrictions imposed on the availability to the public of the deposited materials will  
2 be irrevocably removed upon the granting of a patent.

3 The hybridoma cell line 11BD-2E11-2 was deposited, in accordance with the  
4 Budapest Treaty, with the American Type Culture Collection, 10801 University Blvd.,  
5 Manassas, VA 20110-2209 on November 11, 2003, under Accession Number PTA-5643.  
6 In accordance with CFR 1.808, the depositors assure that all restrictions imposed on the  
7 availability to the public of the deposited materials will be irrevocably removed upon the  
8 granting of a patent.

9 To produce the hybridoma that produce the anti-cancer antibody 7BD-33-11A  
10 single cell suspensions of the antigen, i.e. human breast cancer cells, were prepared in cold  
11 PBS. Eight to nine weeks old BALB/c mice were immunized by injecting 100 microliters  
12 of the antigen-adjuvant containing between 0.2 million and 2.5 million cells in divided  
13 doses both subcutaneously and intraperitoneally with Freund's Complete Adjuvant.  
14 Freshly prepared antigen-adjuvant was used to boost the immunized mice at between 0.2  
15 million and 2.5 million cells in the same fashion three weeks after the initial immunization,  
16 and two weeks after the last boost. A spleen was used for fusion at least two days after the  
17 last immunization. The hybridomas were prepared by fusing the isolated splenocytes with  
18 Sp2/0 myeloma partners. The supernatants from the fusions were tested for subcloning of  
19 the hybridomas.

20 To produce the hybridoma that produce the anti-cancer antibody 1A245.6 single  
21 cell suspensions of the antigen, i.e. human breast cancer cells, were prepared in cold PBS.

1 Eight to nine weeks old BALB/c mice were immunized by injecting 100 microliters of the  
2 antigen-adjuvant containing 2.5 million cells in divided doses both subcutaneously and  
3 intraperitoneally with Freund's Complete Adjuvant. Freshly prepared antigen-adjuvant was  
4 used to boost the immunized mice at 2.5 million cells in the same fashion three weeks after  
5 the initial immunization, two weeks later, five weeks later and three weeks after the last  
6 boost. A spleen was used for fusion at least three days after the last immunization. The  
7 hybridomas were prepared by fusing the isolated splenocytes with NSO-1 myeloma  
8 partners. The supernatants from the fusions were tested for subcloning of the hybridomas.

9 To produce the hybridoma that produce the anti-cancer antibody 11BD-2E11-2  
10 single cell suspensions of the antigen, i.e. human breast cancer cells, were prepared in cold  
11 PBS. Eight to nine weeks old BALB/c mice were immunized by injecting 100 microliters  
12 of the antigen-adjuvant containing between 0.2 million and 2.5 million cells in divided  
13 doses both subcutaneously and intraperitoneally with Freund's Complete Adjuvant.  
14 Freshly prepared antigen-adjuvant was used to boost the immunized mice at between 0.2  
15 million and 2.5 million cells in the same fashion two to three weeks after the initial  
16 immunization, and two weeks after the last boost. A spleen was used for fusion at least  
17 two days after the last immunization. The hybridomas were prepared by fusing the isolated  
18 splenocytes with NSO-1 myeloma partners. The supernatants from the fusions were tested  
19 for subcloning of the hybridomas.

20 To determine whether the antibodies secreted by hybridoma cells are of the IgG or  
21 IgM isotype, an ELISA assay was employed. 100 microliters/well of goat anti-mouse IgG  
22 + IgM (H+L) at a concentration of 2.4 micrograms/mL in coating buffer (0.1M

1 carbonate/bicarbonate buffer, pH 9.2-9.6) at 4°C was added to the ELISA plates overnight.  
2 The plates were washed thrice in washing buffer (PBS + 0.05% Tween). 100  
3 microliters/well blocking buffer (5% milk in wash buffer) was added to the plate for 1 hr.  
4 at room temperature and then washed thrice in washing buffer. 100 microliters/well of  
5 hybridoma supernatant was added and the plate incubated for 1 hr. at room temperature.  
6 The plates were washed thrice with washing buffer and 1/5000 dilution of either goat anti-  
7 mouse IgG or IgM horseradish peroxidase conjugate (diluted in PBS containing 1%  
8 bovine serum albumin), 100 microliters/well, was added. After incubating the plate for 1  
9 hr. at room temperature the plate was washed thrice with washing buffer. 100  
10 microliters/well of TMB solution was incubated for 1-3 minutes at room temperature. The  
11 color reaction was terminated by adding 100 microliters/well 2M H<sub>2</sub>SO<sub>4</sub> and the plate was  
12 read at 450 nm with a Perkin-Elmer HTS7000 plate reader. As indicated in Table 1 the  
13 7BD-33-11A, 1A245.6, 11BD-2E11-2 hybridomas secreted primarily antibodies of the  
14 IgG isotype.

15       After one to four rounds of limiting dilution hybridoma supernatants were tested  
16 for antibodies that bound to target cells in a cell ELISA assay. Three breast cancer cell  
17 lines were tested: MDA-MB-231 (also referred to as MB-231), MDA-MB-468 (also  
18 referred to as MB-468), and SKBR-3. The plated cells were fixed prior to use. The plates  
19 were washed thrice with PBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub> at room temperature. 100  
20 microliters of 2% paraformaldehyde diluted in PBS was added to each well for ten minutes  
21 at room temperature and then discarded. The plates were again washed with PBS  
22 containing MgCl<sub>2</sub> and CaCl<sub>2</sub> three times at room temperature. . Blocking was done with

1 100 microliters/well of 5% milk in wash buffer (PBS + 0.05% Tween) for 1 hr at room  
2 temperature. The plates were washed thrice with wash buffer and the hybridoma  
3 supernatant was added at 100 microliters/well for 1 hr at room temperature. The plates  
4 were washed three times with wash buffer and 100 microliters/well of 1/5000 dilution of  
5 goat anti-mouse IgG or IgM antibody conjugated to horseradish peroxidase (diluted in PBS  
6 containing 1% bovine serum albumin) was added. After a one hour incubation at room  
7 temperature the plates were washed three times with wash buffer and 100 microliter/well  
8 of TMB substrate was incubated for 1-3 minutes at room temperature. The reaction was  
9 terminated with 100 microliters/well 2M H<sub>2</sub>SO<sub>4</sub> and the plate read at 450 nm with a Perkin-  
10 Elmer HTS7000 plate reader. The results as tabulated in Table 1 were expressed as the  
11 number of folds above background compared to the IgG isotype control (3BD-27). The  
12 antibodies from the 7BD-33-11A and 1A245.6 hybridoma cell lines bound strongly to all 3  
13 breast lines, with binding at least 6 times greater than background. Both antibodies bound  
14 most strongly to the MDA-MB-231 cell line. The antibodies from the 11BD-2E11-2  
15 hybridoma cell line also bound most strongly to the MDA-MB-231 cell line, but did not  
16 demonstrate binding on the other 2 cell lines greater than background. These results  
17 suggest that the epitope recognized by this antibody is not present on MDA-MB-468 or  
18 SKBR-3 cells, and is distinct from the epitopes recognized by 7BD-33-11A and 1A245.6.

19 In conjunction with testing for antibody binding the cytotoxic effect of the  
20 hybridoma supernatants were tested in the same breast cancer cell lines: MDA-MB-231,  
21 MDA-MB-468 and SKBR-3. The Live/Dead cytotoxicity assay was obtained from  
22 Molecular Probes (Eu,OR). The assays were performed according to the manufacturer's



1 instructions with the changes outlined below. Cells were plated before the assay at the  
2 predetermined appropriate density. After 2 days, 100 microliters of supernatant from the  
3 hybridoma microtitre plates were transferred to the cell plates and incubated in a 5% CO<sub>2</sub>  
4 incubator for 5 days. The wells that served as the positive controls were aspirated until  
5 empty and 100 microliters of sodium azide and/or cycloheximide was added. 3BD-27  
6 monoclonal antibody was also added as an isotype control since it was known not to bind  
7 to the three breast cancer cell lines being tested. An anti-EGFR antibody (C225) was also  
8 used in the assay for comparison. After 5 days of treatment, the plate was then emptied by  
9 inverting and blotted dry. Room temperature DPBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub> was  
10 dispensed into each well from a multichannel squeeze bottle, tapped three times, emptied  
11 by inversion and then blotted dry. 50 microliters of the fluorescent Live/Dead dye diluted  
12 in DPBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub> was added to each well and incubated at 37°C in a  
13 5% CO<sub>2</sub> incubator for 30 minutes. The plates were read in a Perkin-Elmer HTS7000  
14 fluorescence plate reader and the data was analyzed in Microsoft Excel. The results were  
15 tabulated in Table 1.

16 Differential cytotoxicity was observed with the 3 antibodies. 11BD-2E11-2  
17 demonstrated killing of 39-73%, with the highest cytotoxicity observed in SKBR-3 cells.  
18 1A245.6 and 7BD-33-11A demonstrated similar cytotoxicity in MDA-MB-231 cells, but  
19 1A245.6 was also cytotoxic to MDA-MB-468 cells, while 7BD-33-11A was not.

20 This indicated the antibody derived from the hybridoma cell can produce  
21 cytotoxicity in cancer cells. There was also a general association between the degree of  
22 antibody binding and the cytotoxicity produced by the hybridoma supernatants. There were

1 several exceptions to this trend such as the amount of cytotoxicity produced by 11BD-  
 2 2E11-2 in MB-468 cancer cells, and SKBR-3 cancers despite a paucity of binding. This  
 3 suggested that the antibody has a mediating action that was not detected by the cell ELISA  
 4 binding assay in this cell type, or the assay did not detect the binding, which may be due to  
 5 the constraints of the assay such as cell fixation. Finally, there existed yet another  
 6 possibility, that is, the assay was not sensitive enough to detect the binding that was  
 7 sufficient to mediate cytotoxicity in this particular situation. The other exception was the  
 8 relative paucity of cytotoxicity of 7BD-33-11A towards MB-468 cells despite a 6 fold  
 9 increase in binding over the background in comparison to an isotype control. This pointed  
 10 to the possibility that binding was not necessarily predictive of the outcome of antibody  
 11 ligation of its cognate antigen. The known non-specific cytotoxic agents cycloheximide  
 12 produced cytotoxicity as expected.

13

Table 1	Cytotoxicity (%)						Binding (above bkgd)		
	MB-231		MB-468		SKBR-3		MB-231	MB-468	SKBR-3
	Average	CV	Average	CV	Average	CV	Fold	Fold	Fold
1A245.6	17	7	13	5	44	8	23	10	16
7BD-33-11A	16	2	2	2	29	3	13	6	9
11BD-2E11-2	39	2	66	1	73	18	11	2	1
Cycloheximide	49	9	24	5	56	14			

14

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16

17

1     EXAMPLE 2

2     Antibody Production

3             Monoclonal antibodies were produced by culturing the hybridomas, 7BD-33-11A,  
4     1A245.6, 11BD-2E11-2, in CL-1000 flasks (BD Biosciences, Oakville, ON) with  
5     collections and reseeded occurring twice/week and standard antibody purification  
6     procedures with Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Baie d'Urfé,  
7     QC). It is within the scope of this invention to utilize monoclonal antibodies which are  
8     humanized, chimerized or murine antibodies. 7BD-33-11A, 1A245.6, 11BD-2E11-2 were  
9     compared to a number of both positive (anti-Fas (EOS9.1, IgM, kappa, 20 micrograms/mL,  
10    eBioscience, San Diego, CA), anti-Her2/neu (IgG1, kappa, 10 microgram/mL, Inter  
11    Medico, Markham, ON), anti-EGFR (C225, IgG1, kappa, 5 microgram/mL, Cedarlane,  
12    Hornby, ON), Cycloheximide (100 micromolar, Sigma, Oakville, ON), NaN<sub>3</sub> (0.1%,  
13    Sigma, Oakville, ON)) and negative (107.3 (anti-TNP, IgG1, kappa, 20 microgram/mL,  
14    BD Biosciences, Oakville, ON), G155-178 (anti-TNP, IgG2a, kappa, 20 microgram/mL,  
15    BD Biosciences, Oakville, ON), MPC-11 (antigenic specificity unknown, IgG2b, kappa,  
16    20 microgram/mL), J606 (anti-fructosan, IgG3, kappa, 20 microgram/mL), IgG Buffer  
17    (2%)) controls in a cytotoxicity assay (Table 2). Breast cancer (MB-231, MB-468, MCF-  
18    7), colon cancer (HT-29, SW1116, SW620), lung cancer (NCI H460), ovarian cancer  
19    (OVCAR), prostate cancer (PC-3), and non-cancer (CCD 27sk, Hs888 Lu) cell lines were  
20    tested (all from the ATCC, Manassas, VA). The Live/Dead cytotoxicity assay was  
21    obtained from Molecular Probes (Eugene, OR). The assays were performed according to  
22    the manufacturer's instructions with the changes outlined below. Cells were plated before

1 the assay at the predetermined appropriate density. After 2 days, 100 microliters of  
2 purified antibody was diluted into media, and then were transferred to the cell plates and  
3 incubated in a 8% CO<sub>2</sub> incubator for 5 days. The plate was then emptied by inverting and  
4 blotted dry. Room temperature DPBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub> was dispensed into  
5 each well from a multichannel squeeze bottle, tapped three times, emptied by inversion and  
6 then blotted dry. 50 microliters of the fluorescent Live/Dead dye diluted in DPBS  
7 containing MgCl<sub>2</sub> and CaCl<sub>2</sub> was added to each well and incubated at 37°C. in a 5% CO<sub>2</sub>  
8 incubator for 30 minutes. The plates were read in a Perkin-Elmer HTS7000 fluorescence  
9 plate reader and the data was analyzed in Microsoft Excel and the results were tabulated in  
10 Table 2. The data represented an average of four experiments tested in triplicate and  
11 presented qualitatively in the following fashion: 4/4 experiments greater than threshold  
12 cytotoxicity (+++), 3/4 experiments greater than threshold cytotoxicity (++), 2/4  
13 experiments greater than threshold cytotoxicity (+). Unmarked cells in Table 2 represented  
14 inconsistent or effects less than the threshold cytotoxicity. The 7BD-33-11A and 1A245.6  
15 antibodies demonstrated cytotoxicity in breast and prostate tumor cell lines selectively,  
16 while having no effect on non-transformed normal cells. Both demonstrated a 25-50%  
17 greater killing than the positive control anti-Fas antibody. 11BD-2E11-2 was specifically  
18 cytotoxic in breast and ovarian cancer cells, and did not affect normal cells. The chemical  
19 cytotoxic agents induced their expected cytotoxicity while a number of other antibodies  
20 which were included for comparison also performed as expected given the limitations of  
21 biological cell assays. In total, it was shown that the three antibodies have cytotoxic  
22 activity against a number of cancer cell types. The antibodies were selective in their

1 activity since not all cancer cell types were susceptible. Furthermore, the antibodies  
2 demonstrated functional specificity since they did not produce cytotoxicity against non-  
3 cancer cell types, which is an important factor in a therapeutic situation.

Table 2		BREAST			COLON			LUNG	Ovary	PROSTATE	NORMAL	
		MB-231	MB-468	MCF-7	HT-29	SW1116	SW620	NCI H460	OVCAR	PC-3	CCD 27sk	Hs888 Lu
Positive Controls	11BD2E11-2	-	-	+	-	-	-	-	+	-	-	-
	7BD-33-11A	-	-	+	-	-	-	-	-	++	-	-
	1A245.6	-	-	+	-	-	-	-	-	++	-	-
	anti-Fas	-	-	+++	-	-	-	-	+++	+	-	+
	anti-Her2	+	-	+	-	-	-	-	+	-	-	-
	anti-EGFR	-	+++	+	-	+++	-	-	+	-	+	-
Positive Controls	CHX (100 $\mu$ M)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	NaN <sub>3</sub> (0.1%)	+++	+++	+++	+++	-	-	+++	+++	+++	-	-
Negative Controls	IgG1							+++		+		
	IgG2a			+++		+						
	IgG2b			+++								
	IgG3											
	IgG Buffer	+										

4  
5 Cells were prepared for FACS by initially washing the cell monolayer with DPBS  
6 (without Ca<sup>++</sup> and Mg<sup>++</sup>). Cell dissociation buffer (INVITROGEN) was then used to  
7 dislodge the cells from their cell culture plates at 37°C. After centrifugation and collection  
8 the cells were resuspended in Dulbecco's phosphate buffered saline containing MgCl<sub>2</sub>,  
9 CaCl<sub>2</sub> and 25% fetal bovine serum at 4°C (wash media) and counted, aliquoted to  
10 appropriate cell density, spun down to pellet the cells and resuspended in staining media  
11 (DPBS containing MgCl<sub>2</sub> and CaCl<sub>2</sub>) containing 7BD-33-11A, 1A245.6, 11BD-2E11-2 or  
12 control antibodies (isotype control or anti-EGF-R) at 20 micrograms/mL on ice for 30  
13 minutes. Prior to the addition of Alexa Fluor 488-conjugated secondary antibody the cells  
14 were washed once with wash media. The Alexa Fluor 488-conjugated antibody in staining  
15 media was then added for 20 minutes. The cells were then washed for the final time and

1 resuspended in staining media containing 1 microgram/mL propidium iodide. Flow  
2 cytometric acquisition of the cells was assessed by running samples on a FACScan using  
3 the CellQuest software (BD Biosciences). The forward (FSC) and side scatter (SSC) of the  
4 cells were set by adjusting the voltage and amplitude gains on the FSC and SSC detectors.  
5 The detectors for the three fluorescence channels (FL1, FL2, and FL3) were adjusted by  
6 running cells stained with purified isotype control antibody followed by Alexa Fluor 488-  
7 conjugated secondary antibody such that cells had a uniform peak with a median  
8 fluorescent intensity of approximately 1-5 units. Live cells were acquired by gating for  
9 FSC and propidium iodide exclusion. For each sample, approximately 10,000 live cells  
10 were acquired for analysis and the resulted presented in Table 3. Table 3 tabulated the  
11 mean fluorescence intensity fold increase above isotype control and is presented  
12 qualitatively as: less than 5 (-); 5 to 50 (+); 50 to 100 (++); above 100 (+++) and in  
13 parenthesis, the percentage of cells stained.

Table 3

Antibody	Isotype	BREAST			COLON			LUNG	Ovary	PROSTATE
		MB231	MB468	MDA-7	HT-29	SW610	SW620	NO-H80	OVCAR	PC3
11BD2E11-2	IgG1, k	+(61%)	-	-	-	-	-	-	-	-
7BD33-11A	IgG2a, k	+(98%)	-	+(78%)	+(97%)	+(34%)	+(bimodal, 78%)	+(bimodal, 60%)	+(51%)	+(75%)
1A245.6	IgG1, k	+(98%)	+(78%)	+(74%)	++	+(23%)	+(bimodal, 71%)	+(bimodal, 70%)	+(73%)	+(bimodal, 72%)
anti-EGFR	IgG1, k	++	++bimodal	-	+(97%)	+(43%)	-	+(bimodal, 80%)	+(90%)	+(98%)
anti-FAS	IgM, k	-	-	-	+(30%)	-	-	+(61%)	-	-

15  
16 Representative histograms of 7BD-33-11A antibodies were compiled for Figure 1,  
17 1A245.6 antibodies were compiled for Figure 2, 11BD-2E11-2 were compiled for Figure 3  
18 and evidence the binding characteristics, inclusive of illustrated bimodal peaks, in some

1 cases. 11BD-2E11-2 displayed specific tumor binding to the breast tumor cell line MDA-  
2 MB-231. Both 7BD-33-11A and 1A245.6 displayed similar binding to cancer lines of  
3 breast (MDA-MB-231 and MCF-7); colon, lung, ovary, and prostate origin and differential  
4 binding to one of the breast cancer cell lines (MDA-MB-468). There was binding of all  
5 three antibodies to non-cancer cells, however that binding did not produce cytotoxicity.  
6 This was further evidence that binding was not necessarily predictive of the outcome of  
7 antibody ligation of its cognate antigen, and was a non-obvious finding. This suggested  
8 that the context of antibody ligation in different cells was determinative of cytotoxicity rather  
9 than just antibody binding.

10

### 11 EXAMPLE 3.

#### 12 In vivo experiments

13 Now with reference to the data shown in Figures 5 and 6, four to eight week old,  
14 female SCID mice were implanted with 5 million MDA-MB-231 human breast cancer cells  
15 in one hundred microliters injected subcutaneously in the scruff of the neck. The mice  
16 were randomly divided into four treatment groups of ten. On the day prior to implantation  
17 20 mg/kg of either 11BD2E-11-2, 7BD-33-11A, 1A245.6 test antibodies or 3BD-27  
18 isotype control antibody (known not to bind MDA-MB-231 cells) were administered  
19 intraperitoneally at a volume of 300 microliters after dilution from the stock concentration  
20 with a diluent that contained 2.7 mM KCl, 1 mM  $\text{KH}_2\text{PO}_4$ , 137 mM NaCl, 20 mM  
21  $\text{Na}_2\text{HPO}_4$ . The antibodies were then administered once per week for a period of 7 weeks in  
22 the same fashion.

1 Tumor growth was measured about every seventh day with calipers for up to ten  
2 weeks or until individual animals reached the Canadian Council for Animal Care (CCAC)  
3 end-points. Body weights of the animals were recorded for the duration of the study. At the  
4 end of the study all animals were euthanised according to CCAC guidelines.  
5 There were no clinical signs of toxicity throughout the study. Body weight measured at  
6 weekly intervals was a surrogate for well-being and failure to thrive. There was a minimal  
7 difference in weight for the groups treated with the isotype control, 3BD-27, and 7BD-33-  
8 11A, 1A245.6, or 11BD-2E11-2. At day 60 (11 days after the cessation of treatment)  
9 tumor volume of the group treated with 1A245.6 was 5.2% of the control group  
10 ( $p=0.0002$ ) and demonstrated effectiveness at reducing tumor burden with antibody  
11 treatment. Those mice bearing cancer treated with 7BD-33-11A antibody were disease free  
12 and had no tumor burden. The tumor volume was lower in the 11BD-2E11-2 treatment  
13 group (45% of control) at day 67 ( $p=0.08$ ). This also demonstrated a lesser tumor burden  
14 with cytotoxic antibody treatment in comparison to a control antibody. There was also  
15 corresponding survival benefits (Fig. 6) from treatment with 7BD-33-11A, 1A245.6, and  
16 11BD-2E11-2 cytotoxic antibodies. The control group treated with 3BD-27 antibody  
17 reached 100% mortality by day 74 post-implantation. In contrast, groups treated with  
18 7BD-33-11A were disease free and 1A245.6 treated animal displayed 100% survival and  
19 the group treated with 11BD-2E11-2 had 24% survival.

20 In total, cytotoxic antibody treatment produced a decreased tumor burden and  
21 increased survival in comparison to a control antibody in a well recognized model of  
22 human cancer disease suggesting pharmacologic and pharmaceutical benefits of these



1 antibodies (7BD-33-11A, 1A245.6, 11BD-2E11-2) for therapy in other mammals,  
2 including man.

3

#### 4 EXAMPLE 4.

5 In vivo established tumor experiments

6 Five to six week old, female SCID mice were implanted with 5 million MDA-MB-  
7 231 breast cancer cells in one hundred microliters injected subcutaneously in the scruff of  
8 the neck. Tumor growth was measured with calipers every week. When the majority of the  
9 cohort reached a tumor volume of 100 mm<sup>3</sup> (range 50-200 mm<sup>3</sup>) at 34 days post  
10 implantation 8-10 mice were randomly assigned into each of three treatment groups. 7BD-  
11 33-11A, 1A245.6 test antibodies or 3BD-27 isotype control antibody (known not to bind  
12 MDA-MB-231 cells) were administered intraperitoneally with 15 mg/kg of antibodies at a  
13 volume of 150 microliters after dilution from the stock concentration with a diluent that  
14 contained 2.7 mM KCl, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 20 mM Na<sub>2</sub>HPO<sub>4</sub>. The antibodies  
15 were then administered three times per week for 10 doses in total in the same fashion until  
16 day 56 post-implantation. Tumor growth was measured about every seventh day with  
17 calipers until day 59 post-implantation or until individual animals reached the Canadian  
18 Council for Animal Care (CCAC) end-points. Body weights of the animals were recorded  
19 for the duration of the study. At the end of the study all animals were euthanised according  
20 to CCAC guidelines.

21 There were no clinical signs of toxicity throughout the study. Body weight was  
22 measured at weekly intervals. There was no significant difference in weight for the groups

1 treated with the isotype control and 7BD-33-11A, or 1A245.6 antibodies. As can be seen in  
2 Figure 4, at day 59 post-implantation (2 days after the cessation of treatment), tumor  
3 volume of the group treated with 7BD-33-11A was 29.5% of the control group ( $p=0.0003$ ).  
4 In this group, there was also a trend toward regression in mean tumor volume when the  
5 value for day 59 was compared to day 52 ( $p=0.25$ ). Likewise, treatment with 1A245.6  
6 antibody also significantly suppressed tumor growth and decreased tumor burdens.  
7 Animals with established tumors treated with this antibody had tumor volumes that were  
8 56.3% of the isotype treated control group ( $p=0.017$ ).

9 In total, treatment with 7BD-33-11A or 1A245.6 antibodies significantly decreased  
10 the tumor burden of established tumors in comparison to a control antibody in a well  
11 recognized model of human cancer disease suggesting pharmacologic and pharmaceutical  
12 benefits of these antibodies for therapy in other mammals, including man.

13 All patents and publications mentioned in this specification are indicative of the  
14 levels of those skilled in the art to which the invention pertains. All patents and  
15 publications are herein incorporated by reference to the same extent as if each individual  
16 publication was specifically and individually indicated to be incorporated by reference.  
17 It is to be understood that while a certain form of the invention is illustrated, it is not to be  
18 limited to the specific form or arrangement of parts herein described and shown. It will be  
19 apparent to those skilled in the art that various changes may be made without departing  
20 from the scope of the invention and the invention is not to be considered limited to what is  
21 shown and described in the specification. One skilled in the art will readily appreciate  
22 that the present invention is well adapted to carry out the objects and obtain the ends and

1 advantages mentioned, as well as those inherent therein. Any oligonucleotides, peptides,  
2 polypeptides, biologically related compounds, methods, procedures and techniques  
3 described herein are presently representative of the preferred embodiments, are intended to  
4 be exemplary and are not intended as limitations on the scope. Changes therein and other  
5 uses will occur to those skilled in the art which are encompassed within the spirit of the  
6 invention and are defined by the scope of the appended claims. Although the invention has  
7 been described in connection with specific preferred embodiments, it should be understood  
8 that the invention as claimed should not be unduly limited to such specific embodiments.  
9 Indeed, various modifications of the described modes for carrying out the invention which  
10 are obvious to those skilled in the art are intended to be within the scope of the following  
11 claims.

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